Antibodies

A LABORATORY MANUAL

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Haptens

Many small chemicals can be used to raise antibodies, if they are coupled to larger protein molecules. The small compounds are known as haptens, while the proteins to which they are coupled to are called carriers. The haptens themselves serve as epitopes for binding to the antibodies on the B-cell surface, and the carriers provide the class II–T-cell receptor binding sites. In general, haptens should be coupled to soluble carriers such as bovine serum albumin (BSA) or keyhole limpet hemacyanin (KLH). The coupling mechanism will vary with each hapten, but many of the bifunctional coupling reagents listed in Table 5.5 (p. 130) will be helpful. Also, the techniques on the coupling of synthetic peptides to carriers on p. 78 may be applied. In general, approximately 1 mole of hapten per 50 amino acids of carrier is a reasonable coupling ratio.

Synthetic Peptides

The use of synthetic peptides as immunogens has been an important technique in the elucidation of the properties of an antibody response (e.g., Goebel 1938; Anderer 1963; Anderer and Schlumberger 1965; Sela 1966, 1969; Arnon et al. 1971). Recently, as more DNA sequences

Choosing between Bacterial Expression and Peptides for Immunogen Production

When a cloned DNA sequence in available, antibodies can be prepared either using peptides or bacterially expressed proteins. There are strong proponents for both approaches, both groups citing their experiences favoring one method over the other. Both have advantages and disadvantages, and for a particular antigen one may be better suited than another. However, if both approaches are available to a researcher, both should be used.

For anti-peptide antibodies, a good response to the desired peptide usually can be generated with careful selection of the sequence and coupling method. Because of the way in which the peptide is displayed to the immune system, most peptides elicit a good response. Therefore, the anti-peptide approach has major advantages if the antigen is known to be highly conserved. Likewise, if antibodies need to be raised against a particular region, anti-peptide antibodies have many advantages. The major disadvantage with anti-peptide antibodies is that they may not recognize the native antigen. The percentage of antibodies raised against peptides that will bind to the native protein will vary from antigen to antigen. Values reported in the

and their corresponding protein sequences have become known, synthetic peptides have been used to prepare antibodies specific for previously uncharacterized proteins (Sutcliffe et al. 1980; Walter et al. 1980; and reviewed in Lerner 1982, 1984; Walter 1986; Doolittle 1976; and in Ciba Foundation 1986). Peptides are normally synthesized using the solid-phase techniques pioneered by Merrifield (1963). The synthetic peptides are purified and coupled to carrier proteins, and these conjugates are then used to immunize animals. In these cases, the peptides serve as haptens with the carrier proteins, providing good sites for class II–T-cell receptor binding. Peptide–carrier conjugates seldom fail to elicit a response because of tolerance. Consequently, the peptides can usually be seen as epitopes, and high-titered antisera commonly are prepared. Characteristically, these antibodies will bind well to denatured proteins, but may or may not recognize the native protein.

The two most important advantages of anti-peptide antibodies are that they can be prepared immediately after determining the amino acid sequence of a protein (either from protein sequencing or from DNA sequencing) and that particular regions of a protein can be targeted specifically for antibody production. Rapid conversion from DNA sequence information to antibodies has enormous potential for application in molecular biology. Similarly, the production of site-specific antibodies has immediate implications for functional and clinical studies.

literature range from 0/4 to 3/4 of anti-peptide antibodies will bind to the native antigen. Synthetic peptide antigens are also more expensive to produce than bacterial fusion protein antigens.

Bacterially expressed antigens present a different set of problems. Some will be difficult to express in *E. coli*, presumably because of their toxic side effects. In these cases, inducible systems such as the T7 systems of Studier (see Rosenberg 1987; Studier and Moffatt 1986) are recommended. Even when high levels of the antigen of interest can be produced, there may be some instances where the protein will not be immunogenic or where the antibodies will not recognize the native protein. However, because of the larger size of the bacterially expressed protein, there is a better chance that the antibodies will bind to the native protein.

A reasonable compromise for antibody production would be: (1) If the budget is limited and/or antibodies for the native protein are essential, use fusion proteins or full-length expression in *E. coli*. (2) If the budget is large enough, try both bacterially produced immunogens and peptides. (3) If the protein is highly conserved, use peptides. (4) If site-directed antibodies are needed, use peptides or prepare large banks of monoclonal antibodies against the bacterially produced immunogens.

The major problem that is encountered when preparing anti-peptide antibodies is whether they will recognize the native protein. Assays that need or benefit from anti-native antibodies, such as immunoprecipitation, many cell staining techniques, or immunoaffinity purification, will succeed only when the peptide sequence is displayed on the surface of the native molecule in a conformation similar to the peptide–carrier conjugate. Therefore, the successful production of anti-peptide antibodies is often determined by the researcher's ability to predict the location of certain peptide sequences in the three-dimensional structure of the protein.

Because of their size, peptides may not be immunogenic on their own. To elicit an antibody response directly, they must contain all of the features of any immunogen, notably they must have an epitope for B-cell binding and a site for class II–T-cell receptor binding. Some peptides, even surprisingly small ones, contain both these sites (or more properly, one sequence that can serve both functions), and these peptides can be used without carriers (e.g., see Beachy et al. 1981; Lerner et al. 1981; Dreesman 1982; Jackson 1982; Atassi and Webster 1983; Young et al. 1983). Unfortunately, there are no methods, short of immunization, to test this, and therefore, most peptides are coupled to carrier proteins before injection. An exciting recent development is the use of synthetic class II–T-cell receptor sites synthesized directly with the desired epitope (Francis et al. 1987; see also, Good et al. 1987; Borras-Cuesta et al. 1987; Leclerc et al. 1987). Although there are not enough cases to determine how widely applicable this approach will be, the concept is provocative. With this strategy, the peptide of interest is synthesized as either an amino- or carboxy-terminal extension of a known class II–T-cell receptor site. The synthetic peptide, now containing both sites, is injected without coupling and used to induce an antibody response. The first experiments using this approach look very promising, and this may become an important alternative to coupling with carrier proteins.

Peptides usually are synthesized with an automated machine using solid-phase techniques. The methods for synthesis and purification of the peptide are beyond the scope of this book. However, to judge the success of the coupling reaction and to determine the number of moles of peptide bound to the carrier, a small proportion of the peptide needs to be labeled. This can be done by including a small amount of ^{14}C-labeled amino acid in the synthesis or by iodinating a sample of the peptides on a tyrosine or histidine residue (see p. 324) after the synthesis. A small sample of these iodinated peptides can then be added to the coupling reactions to ascertain the success of the coupling.

During immunization, antibodies to the carrier proteins or the coupling agent will also be produced, and these are normally removed by affinity-purifying the anti-peptide antibodies on a column prepared with conjugates of the peptide and a second carrier molecule. Techniques for affinity purification of the antibodies are described in general on p. 313.

Designing the Peptide

Probably the most frequently asked question concerning synthetic peptides is what sequence should be used for the immunogen (reviewed in Doolittle 1986). Although there is no one correct answer, enough anti-peptide antibodies have been raised to make suggestions for peptide choices. However, preparing anti-peptide antibodies is still an empirical exercise. What works well for one immunogen may fail completely for another.

Choosing the Appropriate Peptide Sequence

With careful synthesis, coupling, and immunizations, most sequences can be used to induce antibodies specific for the peptide itself. When considering which sequence to use, most people actually want to know how likely will it be that the anti-peptide antibodies will recognize the native protein. Early work suggested that peptides containing hydrophilic amino acids (Hopp and Woods 1981, 1983; Kyte and Doolittle 1982) and proline residues were more likely to be exposed on the surface of the native protein than other sequences, and many peptides have been prepared using these criteria. In assessing the value of these criteria, hydrophilicity is required but is not sufficient to predict the surface location of a particular sequence. Many strongly hydrophilic amino acid sequences are buried in water pockets or form inter- or intramolecular bonds and are thus excluded from interactions with anti-native antibodies. Therefore, hydrophilicity can be thought of as required but not sufficient for choosing peptide sequences (see p. 661 for hydrophilicity values). Hydrophilic peptides are also more likely to be soluble for coupling reactions.

The presence of proline residues in synthetic peptides originally was suggested because β-turns often form portions of known epitopes. However, the presence of proline residues in peptides does not have much predictive value when antisera are tested for binding to the surface of native proteins. Although many excellent anti-peptide antisera have been prepared against sequences with proline residues, there is not sufficient evidence to target prolines when designing peptides.

More recently, several workers have noted that carboxy-terminal sequences often are exposed and can be targeted for anti-peptide sequences. Although using carboxy-terminal sequences does not guarantee that the resulting antibodies will recognize the native protein, a surprisingly high percentage will. Similarly, many amino-terminal regions are exposed, and these also may make good targets.

Another potentially useful parameter for selecting peptide sequences is the "mobility" of the amino acid residues. Originally, it was noted that the regions of a protein that become epitopes often have a higher temperature than other regions, as determined by NMR and X-ray structure (Moore and Williams 1980; Robinson et al. 1983; Tainer et al. 1984; Westhof et al. 1984). Higher temperature in crystallography and NMR distinguishes regions that are more mobile from

regions that are more static. These observations have led to the suggestion that stretches of amino acids that are more flexible are more likely to be epitopes. In the preparation of anti-peptide antibodies, when a peptide is coupled to a carrier molecule, it has a different local environment than in the original protein. When choosing a sequence for antibody production, a region of the protein that is more flexible will be more likely over time to form a structure that is similar to the peptide–carrier conjugate. Although the measure of mobility may become a useful criterion for selecting good peptide sequences, it has not been tested in enough detail to determine whether it will have any predictive value.

At present, a reasonable order of suggestions for choosing peptide sequences would be:

1. If possible, use more than one peptide.
2. Use the carboxyl-terminal sequence if it is hydrophilic and if a suitable coupling group is available or can be added.
3. Use the amino-terminal sequence if it is hydrophilic and if a suitable coupling group is available or can be added.
4. Use internal hydrophilic regions; perhaps using longer peptides.

Size of the Peptide

The smallest synthetic peptides that will consistently elicit antibodies that bind to the original protein are 6 residues in length. Responses to smaller peptides are typically weak or will not recognize the protein of interest, either in a native or denatured state. Since epitopes consisting of smaller regions have been reported, the lower limit presumably reflects the difficulty of recognizing the smaller peptides coupled to carriers. With peptides of 6 amino acids or slightly larger, the responses vary. Some will generate good antibodies and some will not. Generally, peptides of approximately 10 residues should be used as a lower limit for coupling.

In the literature two strategies are suggested for peptide length. One school suggests using long peptides (up to 40 amino acids long) to increase the number of possible epitopes, while other authors argue that smaller peptides are adequate and their use ensures that the site-specific character of anti-peptide antibodies is retained. Both strategies have been used successfully. Two important preliminary questions to consider are: (1) Does the anti-peptide serum need to recognize the native protein? If so, use longer peptides or prepare anti-peptide antisera against multiple peptides. (2) How good is your peptide synthesis facility? Peptides over 20 residues in length are increasingly difficult to synthesize, yielding products with inappropriate side reactions. Longer peptides also are more likely to contain residues that make the coupling to carrier molecules more difficult. The correct decison between peptides with 10–15 residues and longer peptides will depend on the experimental design and will normally be a compromise between these factors. The safest choice, but also the most expensive, will be to prepare multiple small peptides of 10–15 amino acids in length from various regions of the sequence.

Coupling Strategy

When choosing the sequence for a synthetic peptide, one factor that often is overlooked is the method of coupling. Most coupling methods rely on the presence of free amino, sulfhydryl, phenolic, or carboxylic acid groups. Free amino groups used for coupling will be found on lysine side chains or on the amino-terminal residue. Sulfhydryl groups are found on cysteine side chains, phenolic groups on tyrosines, and carboxylic acid groups on aspartic acids, glutamic acids, and the carboxy-terminal residue. Coupling methods should be used that link the peptide to the carrier via either the carboxy- or amino-terminal residue. When preparing antibodies against the carboxy-terminal region of the protein, the coupling should be done through the amino terminus of the peptide. Similarly, the coupling for amino terminal fragments should be done through the carboxy-terminal region of the peptide. For internal fragments, the major consideration is that the peptide be coupled by an end and not through a central residue.

The easiest strategy to manipulate the type of coupling is to add an extra amino acid on either the amino or carboxyl terminus to allow simple, one-site coupling to the carrier. Any coupling method that potentially can bind to an internal residue should be avoided. Similarly, coupling methods should be chosen that will bind to only one amino acid, if possible. If multiple coupling sites are possible, they should be localized to either the amino or carboxyl terminus, and the coupling should be adjusted to link only through one site per peptide on average. It is important to remember that it is often easier to use different peptides than design elaborate coupling schemes.

Choosing the Appropriate Carrier

Many different carrier proteins can be used for coupling with synthetic peptides. The two most commonly used are keyhole limpet hemacyanin (KLH) and bovine serum albumin (BSA). Both work well in most cases, but each has disadvantages. Because of its large size, KLH is more likely to precipitate during cross-linking, and this can make handling KLH difficult in some cases. On the other hand, BSA is very soluble, but often is a good immunogen in its own right. For most purposes, either carrier will be adequate. Use whichever is more convenient.

Three other carriers that are used occasionally are ovalbumin, mouse serum albumin, or rabbit serum albumin. Ovalbumin can be used as a good carrier for most purposes. It is also a good choice for a second carrier when checking that antibodies are specific for the peptide itself and not the carrier. MSA or RSA may be used when the antibody response to the carrier molecule must be kept to a minimum.

BSA has 59 lysine (30–35 are available for coupling), 19 tyrosine, 35 cysteine, 39 aspartic acid, and 59 glutamic acid residues. Ovalbumin has 20 lysine, 10 tyrosine, 6 cysteine, 14 aspartic acid, and 33 glutamic acid residues.

COUPLING PEPTIDES TO CARRIER PROTEINS USING GLUTARALDEHYDE*

Glutaraldehyde is a bifunctional coupling reagent that links two compounds primarily through their amino groups. Glutaraldehyde cross-linking is one of the most stable linkages used; however, the glutaraldehyde bridge will often form a portion of an epitope recognized by the immunized animal. Positive sera should always be screened in assays using a second glutaraldehyde-coupled peptide. In some cases antisera may be judged mistakenly as positive for the peptide when they react primarily against the glutaraldehyde.

Glutaraldehyde cross-linking is particularly useful when the synthetic peptide contains only a single free amino group at its amino terminus. When peptides with more than one free amino group are used, large multimeric complexes can be formed. In these cases other cross-linking reagents should be considered before using glutaraldehyde. If glutaraldehyde must be used, the ratios between the synthetic peptide and the carrier protein need to adjusted carefully to minimize overcoupling. Also, lowering the pH well below the pK of the amino group (p. 660) will slow the rate of coupling, as the NH_2 group is the target and not NH_3. Other amino acids such as cysteine are also linked with glutaraldehyde, although at a considerably lower frequency.

Two approaches can be used to couple the peptide to the carrier. In the single-step coupling method, the peptide and carrier are mixed at the appropriate ratios and a limiting amount of glutaraldehyde is added. In this case overcoupling is limited by the amount of glutaraldehyde. This method is recommended when more than one free amino group is found on the peptide.

If the peptide is suitably large and contains only one free amino group, a two-step method offers many advantages. The peptide is treated with an excess of glutaraldehyde and then separated from the free glutaraldehyde. By having the coupling agent present only on the peptide, the number of moles of peptide bound to the carrier is easy to control. Also, the carrier molecules cannot be cross-linked to one another. If this approach is used, the glutaraldehyde should be added to the peptide in a large molar excess. This will lower the number of peptide-to-peptide coupling events.

As a general starting point, for each 50 amino acids in the carrier protein, 1–2 moles of peptide are added to the coupling reaction.

*Reichlin (1980).

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